LEUKOCYTE-DEPENDENT HISTAMINE RELEASE FROM RABBIT PLATELETS

The Role of IgE, Basophils, and a Platelet-Activating Factor*

By JACQUES BENVENISTE,‡§ PETER M. HENSON,‖ and CHARLES G. COCHRANE

(From the Department of Experimental Pathology, Scripps Clinic and Research Foundation, La Jolla, California 92037)

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Several immunologic mechanisms have been described which induce the release of vasoactive constituents from platelets (1, 2). Among these is the leukocyte-dependent histamine release (LDHR)[1] mechanism which requires leukocytes but apparently not complement (3–7). To demonstrate the LDHR reaction, washed leukocytes from immunized rabbits are combined with specific antigen and platelets. The platelets aggregate and release their histamine. A soluble factor has been described which is released from the leukocytes and causes aggregation of platelets and release of histamine (7–9). The presence of the LDHR mechanism correlated with the deposition of circulating immune complexes in arteries and glomeruli in acute immune complex disease of rabbits, suggesting that LDHR may be of importance in the pathogenesis of some immunologic diseases (10).

Antigen-induced histamine release from platelets was initially described in rabbits

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‡ Recipient of a fellowship grant from International Agency for Research in Cancer.

§ Present address: Unite de Recherches Nephrologiques de L'Inserm U 25, Hopital Necker, Paris 15e, France.

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[1] *Abbreviations used in this paper:* ACD, acid citrate dextrose; BSA, bovine serum albumin; DFP, diisopropylfluorophosphate; HGG, human gamma globulin; HRP, horseradish peroxidase; LDHR, leukocyte-dependent histamine release; PAF, platelet-activating factor; PAF-BSA, PAF bound to BSA; PBS, phosphate-buffered saline; PCA, passive cutaneous anaphylaxis; SRS-A, slow reacting substance of anaphylaxis; TG, Tyrode's with 0.25% gelatin; TG no Ca^{++}, Tyrode's with 0.25% gelatin from which Ca^{++} has been omitted; TTBSA, Tyrode's with 1×10^{-2} Tris buffer used instead of bicarbonate and 0.25% BSA instead of gelatin; TTBSA no Ca^{++} no Mg^{++}, same medium from which Ca^{++} and Mg^{++} have been omitted. Tyrode's buffer composition is: KCl, 2.6×10^{-3} M; $MgCl_2 \cdot 6H_2O$, 1×10^{-3} M; NaCl, 1.37×10^{-1} M; $NaHCO_3$, 1.2×10^{-2} M; $CaCl_2 \cdot 6H_2O$, 1.3×10^{-3} M. When noted, 2×10^{-4} M EGTA or EDTA were added to TG no Ca^{++}. Unless noted, all solutions were buffered at pH 7.3 at room temperature.

immunized with dinitrophenylated bovine gamma globulin and serum albumin (3). It was then related to the presence of leukocytes and found in rabbits infected with *Schistosoma mansoni* (4) or immunized with various antigens (5–7). LDHR has been correlated with homocytotropic antibody in the serum of immunized rabbits (3, 5, 11). Recently it has been possible to transfer LDHR to leukocytes of normal rabbits in vivo using serum from sensitized animals (12, 13). Speculation as to the nature of the leukocyte involved in LDHR has led to successive proposals of lymphocytes (4, 14) and monocytes (7); however, recent correlative studies suggest that the sensitized cell is the basophil (15).

The present studies were undertaken to gain further information concerning the mechanism of the LDHR reaction: the class of antibody involved, the type of leukocyte, and the characteristics of the soluble platelet-activating factor (PAF) that is released from the sensitized leukocytes upon addition of antigen. The results have indicated that (*a*) the antibody capable of conferring LDHR sensitivity on normal rabbit leukocytes is an IgE antibody, (*b*) the leukocyte involved is a basophil as evidenced by electron microscopy, and (*c*) recovery and stability of the PAF depend on the environment in which the antigen-basophil reaction takes place. We are presenting various technical improvements which have made possible additional analysis of PAF and some of the important factors in immunization of rabbits that have allowed us to obtain consistently strong LDHR responses.

Materials and Methods

Siliconized glassware and needles or plastic materials were used for all cell handling.

Platelets.—Adult New Zealand white rabbits were bled from the ear artery into acid citrate dextrose (ACD) (16) or 5×10^{-3} M ethylenediaminetetraacetate (EDTA), pH 7.2, and platelets were obtained by a modification of the method of Ardlie et al. (17). Blood centrifuged at room temperature at 400 *g* for 20 min separated into three layers: the platelet-rich plasma layer, which was removed; the buffy coat layer, which was processed as described below; and the erythrocyte and neutrophil layer, which was discarded. The platelets were removed from the platelet-rich plasma by centrifugation at 2000 *g* for 20 min, washed twice with Tyrode's with 0.25% gelatin from which Ca^{++} had been omitted (TG no Ca^{++}) and [ethylene bis(oxyethylenenitrilo)]tetraacetic acid (EGTA) pH 6.5 and finally suspended in the same medium. Concentrations of platelets were assessed by optical density at 530 nm, using a standard curve as reference. Rabbit platelets prepared and maintained in this medium at room temperature up to 12 hr could release 90% of their histamine under suitable stimulation in Tyrode's with 2–5% nonspecific release.

Leukocytes.—The buffy coat layer (containing primarily leukocytes and some contaminating platelets and erythrocytes) was removed with an L-tipped Pasteur pipette and suspended in 2.5% gelatin in phosphate-buffered saline (PBS). Erythrocytes were allowed to sediment 30 min at room temperature. The leukocyte-rich supernatant was removed, centrifuged at 450 *g* for 15 min, and the pellet was resuspended in Tyrode's with 1×10^{-2} Tris buffer instead of bicarbonate and 0.25% BSA instead of gelatin (TTBSA). The remaining erythrocytes were lysed by osmotic shock (18) or 0.83% ammonium chloride. Contaminating platelets were removed from the leukocyte suspension either by four to five washes followed by centrifugation for 8 min at 150 *g* or by aggregation with 50 milliunits of thrombin/ml followed by two washes in TTBSA with Ca^{++} and Mg^{++} omitted (TTBSA no Ca^{++} no Mg^{++}). When leukocytes were

used for electron microscopy, the thrombin step was omitted and cells were simply washed twice in TG no Ca^{++} or TTBSA no Ca^{++} no Mg^{++} after the lysis of erythrocytes.

Active Immunization.—Rabbits were immunized with horseradish peroxidase (HRP) (type II, Sigma Chemical Co., St. Louis, Mo.). After testing several schedules, the course chosen involved subcutaneous injection of 10 mg peroxidase in saline or in complete Freund's adjuvant with a subsequent injection of 5 mg peroxidase in saline 3 wk later. Bleeding took place 1–4 wk after the second injection. No differences were noted when the first injection of peroxidase was in complete Freund's adjuvant rather than in saline.

Passive Sensitization.—Sera from actively immunized rabbits were administered intravenously or intraperitoneally to young, nonimmunized rabbits weighing 1.5 kg. The screening tests for LDHR activity (see below) were performed at 24 hr, 48 hr, and 1 wk after transfer of sera. The same schedule was used with fractions of antisera, or with antisera previously heated 2 hr at 56°C or absorbed with goat anti-rabbit γ or goat anti-rabbit ϵ antisera.

Fractionation of Antibody.—Plasma from 10 immunized rabbits was pooled. Globulins obtained by precipitation with 50% ammonium sulfate were fractionated either by ion exchange chromatography on diethylaminoethyl (DEAE)-Sephadex A-50, or by gel filtration chromatography on Sephadex G-200. All fractions were measured for (*a*) absorbance at 280 nm, (*b*) capability of passive sensitization of normal rabbits, (*c*) presence of reaginic antibodies by homologous passive cutaneous anaphylaxis (PCA) (19, 20), and (*d*) estimation of the presence of precipitating antibody by immunoelectrophoresis.

Immunoelectrophoresis.—Immunoelectrophoresis was performed on fractions eluted from the chromatography columns. Precipitating lines were revealed with sheep anti-normal rabbit serum or goat anti-rabbit ϵ-chain. The slides were then washed in PBS for 48 hr, exposed to 1 μg/ml of peroxidase in PBS for 24 hr, and washed 4 days in PBS with frequent buffer changes. Slides were then stained for antibody-bound peroxidase (21) by exposure to 0.01% hydrogen peroxide in 0.05 Tris-HCl buffer at pH 7.5 containing 0.3 mg/ml of 3–3′ diaminobenzidine (tetrahydrochloride) for 30 min at room temperature and subsequently washed with three changes of buffers.

LDHR Test for Screening of Rabbits.—The total formed elements from 2 ml blood collected into 0.01 M EDTA were washed at 4°C with TG no Ca^{++} EGTA, pH 6.5, then with the same medium without EGTA. Cells were divided into two equal portions and diluted to 2.5 ml with TG, pH 7.2. After addition of 50 μg peroxidase to one tube and 20 μg human gamma globulin (HGG) (a noncross-reactive protein) to the other, both were incubated for 30 min at 37°C with mild agitation. After centrifugation the supernatants and the boiled sediments were assayed for histamine on guinea pig ileum by comparison with known dilutions of histamine, and percentage released was calculated.

Platelet-Activating Factor (PAF) Bound to Bovine Serum Albumin (PAF-BSA).—PAF-BSA was obtained by incubation of 1×10^7 platelet-free leukocytes from immunized rabbits at 37°C for 10 min in 1 ml of Tris-Tyrode BSA, with 50 μg of peroxidase. A noncross-reactive antigen (HGG, 20 μg) was used as a control. Cells were removed by centrifugation; the supernatant was dialyzed for 24 hr with many changes of buffer to remove adenosine diphosphate and histamine, and was checked for the presence of PAF-BSA as described below. In some experiments goat anti-ϵ- or goat anti-γ-chain antisera were substituted for peroxidase to release PAF-BSA from sensitized or normal rabbit cells.

Aggregation of Platelets.—Platelet aggregation was examined in an aggregrometer apparatus (Chrono-Log Corp., Broomall, Pa.). $3–5 \times 10^8$ platelets were stirred at 900 rpm at 37°C in 2.5 ml of TG, and the amount of light transmitted through the cell suspension was recorded. After 30 min, the solution was tested for released histamine, then boiled for 2 min, measured again for total histamine, and percentage released was calculated. This system was used to determine the aggregating effect of platelets of (*a*) leukocytes obtained from sensitized or normal rabbits upon addition of peroxidase or anti-immunoglobulin antiserum, or (*b*) various amounts of PAF-BSA produced under the conditions mentioned above.

Inhibition and Induction Experiments.—Inhibition of LDHR was studied by addition of goat anti-rabbit ϵ-chain antiserum[2] or goat anti-rabbit γ-chain antiserum[3] to a given number of leukocytes (between 2.5×10^6 and 1×10^7) in a final volume of 1 ml of TTBSA. The cell suspension was incubated for 5 min at 37°C and centrifuged. The supernatant was discarded, and the cells were resuspended in aggregrometer tubes in 2.5 ml of TG with 2.5×10^8 platelets and antigen (peroxidase). LDHR induction studies were performed by combining leukocytes, platelets, and anti-IgE or anti-IgG antiserum in the aggregrometer tubes.

Cytological Studies.—

Light microscopy: Basophil degranulation was studied by staining suspensions of leukocytes after exposure to peroxidase, or as a control, to nonrelated protein for 10 min at 37°C. Staining was performed by addition to the leukocyte suspension in TTBSA of an equal volume of 0.05% toluidine blue in 95% ethyl alcohol.

Electron microscopy: Studies were performed with two techniques:

(*a*) Leukocytes were suspended in TG or TTBSA at 37°C, then peroxidase or a nonrelated protein was added. Reactions were stopped at 1.0, 2.5, and 5.0 min by addition of prewarmed glutaraldehyde in phosphate or cacodylate buffer, 0.15 M, pH 7.4, to a final concentration of 3% v/v. After 2 hr, the cells were centrifuged, washed in phosphate buffer, fixed in 1% osmium tetroxide in 0.15 M phosphate buffer, pH 7.3, for 2 hr, and embedded in Vestopal. Sections were cut, stained with uranyl acetate and lead citrate, and examined in the Hitachi HU11A microscope (Hitachi Ltd., Tokyo, Japan).

(*b*) Leukocytes and platelets were prepared by the same technique used for the LDHR screening test, except that 15 ml of buffy coat replaced blood as the starting material. After the second wash in TG no Ca^{++}, the white layer on the top of the red cells was gently stirred with a glass rod to ensure mixing of the platelet and leukocyte layers. Brief centrifugation (2–4 sec at 2000 *g*) resulted in a dense layer of mixed platelets and leukocytes. Then Tyrode's containing calcium in excess was added with great care so as not to resuspend the cells. The tubes were warmed to 37°C, peroxidase or a nonrelated protein was added, and the tubes allowed to incubate for periods of time between 1 and 15 min. The supernatants were removed and saved for measure of histamine content, and 5% glutaraldehyde in 0.15 M phosphate buffer, pH 7.3, was gently added on top of the buffy coat layer. The tubes remained 2 hr at room temperature, permitting the white cells to form a gelified disk which was then removed from the tube. This technique was designed so that the spatial relationship between leukocytes and platelets would not be disturbed, and the cells could consequently be fixed and examined by electron microscopy in the same position as that in which they had reacted. Electron microscopy was then carried out as described above.

RESULTS

The LDHR Mechanism in Actively and Passively Immunized Sensitized Rabbits.—Table I shows the results of primary and secondary immunization of rabbits with horseradish peroxidase. Buffy coat cells from donors were not sufficiently sensitized at 1 wk after primary immunization to induce the leukocyte-dependent mechanism of histamine release (LDHR) from platelets.

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[2] This antiserum was generously supplied to us by Dr. Nathan Zvaifler.

[3] Because of the lack of purified rabbit IgE, we could not quantitate the amount of antibody present in the anti-ϵ-chain antiserum in order to compare anti-ϵ-chain and anti-γ-chain antisera. 0.05–0.5 μl of anti-IgG antiserum per 1×10^7 leukocytes induced or inhibited the LDHR reaction, because of anti-light chain activity. After absorption with $F(ab)^2$ fragment of rabbit IgG, the antiserum was no longer active, although immunodiffusion had shown that it retained its anti-γ reactivity. Volumes up to 250 μl were then used for comparison with the anti-ϵ-chain antiserum.

TABLE I

Presence in Immunized Rabbits of LDHR Reaction and of Serum Antibody Capable of Transferring LDHR to Normal Rabbits

	LDHR reactions* % Histamine release‡		
	Donors	Recipients	
		48 hr	7 days
Weeks after first injection of antigen§			
1	<10	0	0
2	44	<10	0
3	76	40	0
4	68	<10	0
5	66	<10	0
6	64	<10	0
Weeks after second injection of antigen§			
100	100	100	60
2	100	100	56
3	100	100	55
4	100	100	61

* At each time blood was taken from one donor; 2 ml was used to determine the LDHR of donors and 30 ml of serum was injected into recipients. LDHR of recipients was measured 24 and 48 hr after transfer.

‡ Per cent of total histamine content of 2-ml samples of blood incubated at 37°C for 30 min with antigen. Values of control preparations without antigen were less than 10%. Each value represents one individual animal. This experiment was done four times with similar results.

§ First injection, 10 mg peroxidase subcutaneously. Second injection (5 mg peroxidase subcutaneously), 3 wk after first.

From 1–6 wk, the levels of LDHR reactivity of the recipients remained low, indicating the presence in donor sera of low levels of antibody capable, upon transfer, of conferring an LDHR reaction to a normal recipient rabbit. By contrast, if given a second injection of antigen 3 wk after the first, all animals presented a strong LDHR response within 1 wk (with 100% histamine release from platelets), reflecting a high level of sensitization of circulating leukocytes. Upon transfer of sera from these immunized rabbits to normal rabbits, all recipients had high levels of LDHR activity reflecting a large amount of transferable antibody in the donors' sera.

Under the conditions of immunization, transfer of 7.5–30 ml of donor serum to nonimmunized rabbits resulted in their passive sensitization after 24 and 48 hr. (No passive sensitization was observed at time periods of less than 12 hr after the injection of serum.) The screening test showed 100% histamine release. 1 wk later the reaction was weak when 7.5 ml was transferred, but clearly detectable after transfer of 15 and 30 ml.

Chromatographic Characterization of the LDHR Antibody.—A 50% ammonium sulfate cut of antiserum from rabbits bled 1–4 wk after the second injection of

peroxidase was chromatographed on a column of DEAE-Sephadex. A typical experiment demonstrating the distribution of the antibody capable of transferring LDHR sensitivity to normal rabbits is shown in Fig. 1. As noted, antibody capable of transferring the LDHR eluted at an ionic strength of 0.13–0.23. This corresponded to the fast γ-globulin region and coincided with the elution of antibody capable of inducing PCA in recipient rabbits. By contrast, antibody to peroxidase was found throughout the elution profile.

Immunoelectrophoretic patterns of the eluates are shown in Fig. 2. When normal recipients were injected with slow γ (PCA negative) fractions, they remained LDHR negative and their sera exhibited anti-peroxidase antibody in the slow γ region. When normal recipients were injected with fast γ fractions

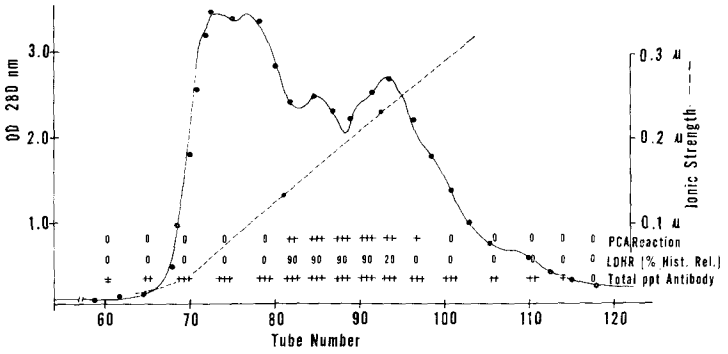


FIG. 1. Chromatographic separation on DEAE-Sephadex A-50 of the globulin fraction of serum from rabbits immunized with peroxidase. Starting buffer was phosphate 0.01 M, pH 8.0. A gradient of NaCl to 0.5 M was started at tube 65. Eluted fractions were tested for the presence of reaginic antibody by passive cutaneous anaphylaxis, its ability to transfer LDHR passively to a normal rabbit, and for the presence of precipitating antibody by immunoelectrophoresis.

eluting between 0.13 and 0.23 ionic strength, and PCA positive, their sera showed a fast γ anti-peroxidase activity. These recipients became LDHR positive. When fractions eluted at ionic strength greater than 0.23 were injected into normal rabbits, sera from the recipients showed a fast γ anti-peroxidase activity but the animals were LDHR negative. No PCA activity was found in these fractions.

When 50% ammonium sulfate cut of antisera from immunized rabbits was fractionated by filtration on G-200 Sephadex, the PCA and LDHR antibody eluted together before IgG immunoglobulins in a region corresponding to 200,000 mol wt (Fig. 3). No PCA or LDHR antibodies were found in the 900,000 mol wt peak nor in the trailing shoulder of the 150,000 mol wt peak.

Inhibition of Transfer of LDHR by Treatment of Antisera with Anti-ϵ-Chain Antiserum or Heating.—Antisera from sensitized rabbits that were known to possess LDHR antibody were treated with anti-ϵ- or anti-γ-chain antisera or

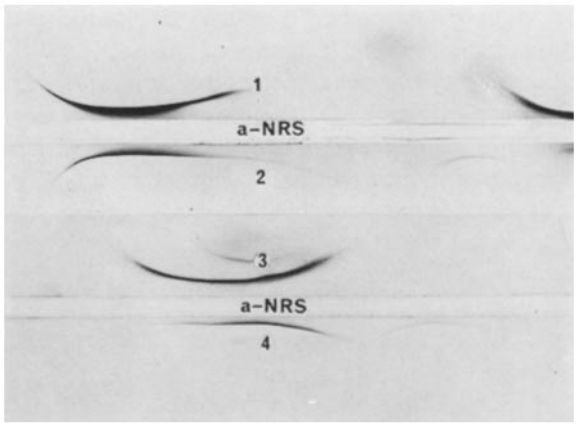


FIG. 2. Enzymatic detection of anti-peroxidase antibody in immunoelectrophoresis in agar The anode is to the right. Top slide well 1: pool 71–72 from the DEAE-Sephadex column shown in Fig. 1. Well 2: serum from recipient 24 hr after injection of pool 71–72 (recipient was LDHR negative). Bottom slide shows well 3, pool 89–90; well 4, serum from recipient 24 hr after injection of pool 89–90 (recipient was LDHR positive). The anti-normal rabbit serum antiserum (a-NRS) was without detectable anti-IgE activity. The peroxidase activity appears as a heavy dark line on this picture (originally of brown color).

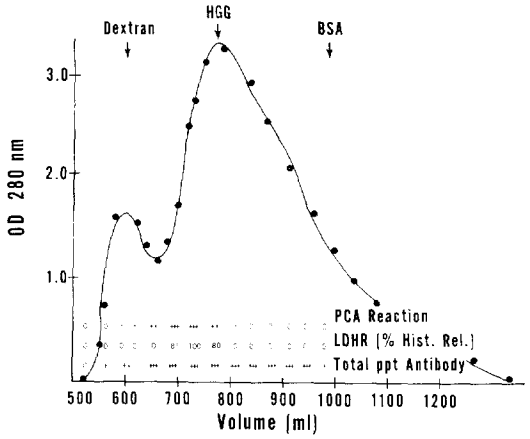


FIG. 3. Gel filtration chromatography of the globulin fraction of serum from rabbits immunized with peroxidase on Sephadex G-200 equilibrated with PBS at pH 7.2. Blue dextran, radiolabeled HGG and BSA, and cytochrome *c* were used as calibrating markers. The assay procedures were the same as those described in the legend to Fig. 1.

were heated at 56°C for 2 hr before transfer to normal recipients. The data, shown in Table II, reveal that treatment with anti-ϵ-chain antiserum or heating removed the ability of the antisera to convey LDHR and PCA reactivity to normal rabbits. Anti-γ-chain antiserum was weakly active in this

respect, which may be explained by some contaminating anti-ε-chain or remaining anti-light chain activity.

Inhibition and Induction of LDHR with Anti-ε-Chain Antiserum.—Preincubation of 1×10^7 sensitized leukocytes in 1 ml TTBSA with 25 μl of anti-ε-chain antiserum abolished completely the ability of the cells to react subsequently with platelets upon addition of antigen (Fig. 4). Neither aggregation

TABLE II

Effect of Anti-ε, Anti-γ Antisera and Heating on the Ability of Serum from Immunized Rabbits to Transfer LDHR and Cutaneous Anaphylaxis to Normal Recipient Rabbit

Amount of serum transferred	Absorbed with*	LDHR of recipients‡		Skin test of recipients 48 hr
		48 hr	7 days	
ml				
30	—	91	89	+
30 heated§		43	<10	ND‖
30	Anti-ε	15	<10	−
7.5	—	90	45	+
7.5	Anti-γ	47	30	+
7.5	Anti-ε	13	<10	−

* 0.5 ml of anti-γ or anti-ε antisera per 15 ml of serum incubated 1 hr at 37°C and overnight at 4°C. This treatment was repeated once. Antisera were made specific for Fc fragment by absorption with $F(ab')_2$.

‡ Per cent of total histamine content of 2 ml samples of blood incubated at 37°C for 30 min with antigen. Values of control preparations without antigen were less than 10%. Each value represents one individual animal. This experiment was done four times with similar results.

§ 56°C for 2 hr.

‖ ND = not done.

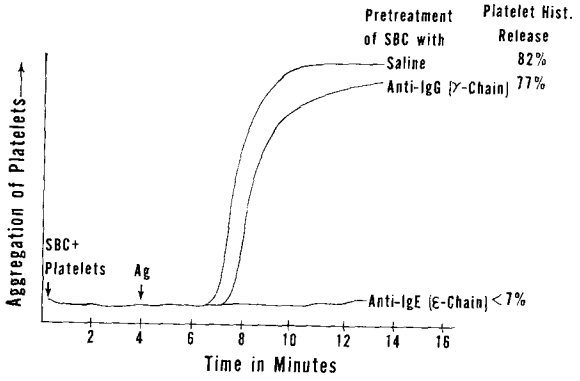


FIG. 4. Inhibition of LDHR by preincubation of sensitized buffy coat cells with anti-IgE (ε-chain) antiserum. Sensitized buffy coat cells (SBC) were incubated in TTBSA containing saline, 250 μl of goat anti-rabbit γ-chain antiserum, or 25 μl goat anti-rabbit ε-chain antiserum for 5 min at 37°C, washed once, and added to platelets in TG in the aggregrometer tube at 37°C with stirring at 900 rpm. Upon addition of peroxidase, variations in optical density were recorded. Ascending lines reflected platelet aggregation as a result of decrease in optical density. Tubes were kept a total of 30 min at 37°C and histamine release was assayed.

nor histamine release was found. Anti-γ-chain antiserum was inactive in this regard when added to the leukocytes even in great excess (250 μl).

When 0.5–50 μl of anti-ε-chain antiserum were added to various amounts of leukocytes in the presence of platelets in the aggregrometer, induction of LDHR occurred as evidenced by aggregation of platelets and release of histamine (Fig. 5). This reaction was identical with that obtained when peroxidase was added to sensitized buffy coat cells and platelets. 250 μl of anti-γ-chain antiserum was inactive in this regard. Buffy coat cells and platelets without anti-ε-chain a ntiserum, or platelets with anti-ε-chain antiserum but without buffy coat cells, showed no aggregation or histamine release above 7%. The number of leukocytes necessary to aggregate 2.5 × 10^8 platelets varied from one ex-

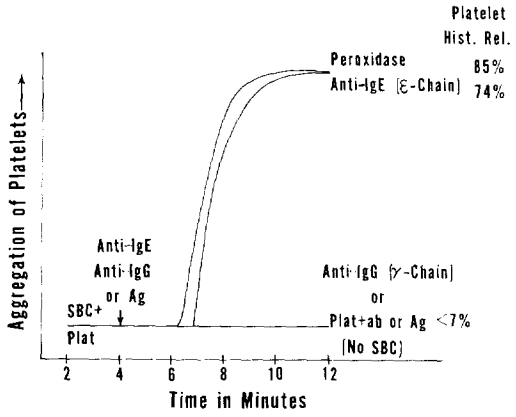


FIG. 5. Aggregation of rabbit platelets during LDHR reaction produced upon addition of peroxidase or goat anti-rabbit ε-chain to sensitized buffy coat cells and platelets mixed together in the aggregrometer tube.

periment to another. Often buffy coat cells from hypersensitized rabbits reacted more strongly (i.e., two to three times fewer cells were necessary) with anti-ε-chain antiserum than did buffy coat cells from nonsensitized rabbits.

Conditions Required for Release and Preservation of PAF. The Association of PAF and Serum Albumins.—When sensitized buffy coat cells were reacted with the specific antigen, they released PAF which could be detected in the cell-free supernatant. Table III shows that to recover PAF, albumin had to be included in the medium. Ovalbumin, gelatin, HGG, peroxidase, and ferritin did not preserve PAF. If platelets or neutrophils were present, detectable release of PAF was diminished. By performing the reaction in the presence of 10^{-3} M diisopropylfluorophosphate (DFP), in the absence of Ca^{++}, or at 4°C, release was inhibited completely.

Several experiments suggested that PAF is actually linked to or closely associated with BSA present in the medium during release of PAF from the

leukocytes. (*a*) PAF eluted from G-200 column and DEAE-Sephadex columns with BSA (Figs. 6 and 7). (*b*) Rabbit anti-BSA antiserum was added to PAF-BSA, and the precipitate was removed by centrifugation. Whereas no PAF activity was found in the supernatant, about 50% of the PAF activity was

TABLE III

*Release of Platelet-Activating Factor (PAF) from Sensitized Leukocytes upon Addition of Antigen in Tyrode's Solution**

Carrier	Inhibitors	PAF units‡ in supernatant
Serum albumin:§ (human, bovine, rabbit, or rat)	—	40
Ovalbumin, HGG, peroxidase ferritin, gelatin	—	0
BSA	Platelets (1×10^9)	6
BSA	Neutrophils (1×10^7)	6
BSA	DFP 10^{-3} M	0
BSA	EDTA-EGTA 10^{-4} M	0
BSA	4–22°C	0

* 1×10^7 leukocytes were reacted in 1 ml of Tyrode's for 10 min at 37°C. Cells were centrifuged, the supernatant dialyzed 24 hr with frequent changes of PBS, and assayed for PAF in the presence of 1.25 mg/ml of apyrase.

‡ PAF units were defined as the reciprocal of the volume in milliliters of PAF necessary to release 50% of histamine from 2.5×10^8 platelets in 2 ml of Tyrode's.

§ All proteins were added to Tyrode's at a concentration of 2.5 mg/ml.

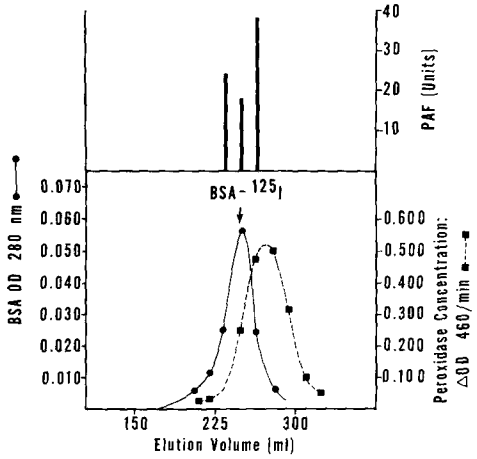


FIG. 6. Elution pattern of PAF-BSA from a Sephadex G-200 gel filtration column equilibrated with PBS at pH 7.2. The buffer in which peroxidase was added to the leukocyte preparation contained BSA-^{125}I. Peroxidase concentration was measured by enzymatic reaction according to reference 22. Other calibrating markers were blue dextran and cytochrome *c*. PAF units were defined as the reciprocal of the amount in milliliters of PAF solution which caused 50% histamine release from 3×10^8 platelets at 37°C in 2 ml of TG.

recovered from the insoluble sediment. PAF induced platelet aggregation and release of histamine with a very short latency period (15–30 sec) (Fig. 8) as compared with the typical LDHR reaction (Fig. 5). More PAF was released from sensitized leukocytes with HRP as compared with anti-ϵ-chain antiserum.

Cytological Studies. Degranulation of Basophils.—Basophils present in the leukocyte preparation were counted after exposure to a nonrelated antigen. As compared with this original population of cells with metachromatic granules, only 6% of them were detectable after exposure to peroxidase and 10% after exposure to anti-ϵ-chain antiserum (Fig. 9). Most of these remaining cells were partially degranulated.

Electron Microscope Studies.—Normal basophils were identified on electron

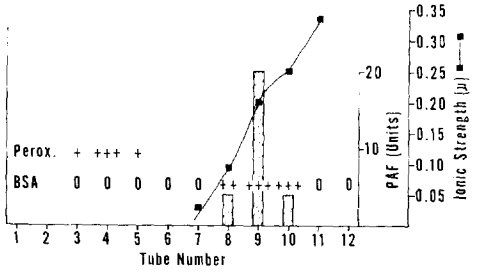


FIG. 7. Elution pattern of PAF-BSA from a DEAE-Sephadex A-50 column. Starting buffer was phosphate 0.01 M, pH 8.0. A gradient of NaCl to 0.5 M was started at tube 7. Peroxidase was detected by a nonquantitative method derived from reference 21 and BSA by precipitation with monospecific anti-BSA antibody.

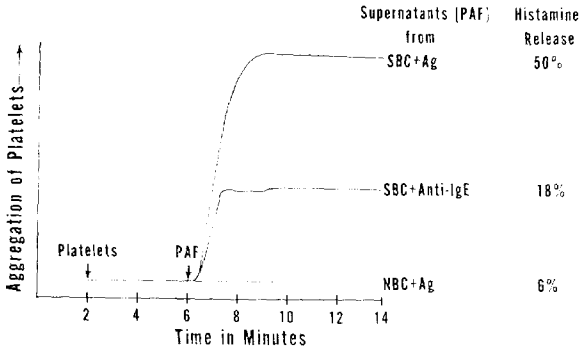


FIG. 8. Aggregation of platelet by platelet-activating factor (PAF). Peroxidase (Ag) or anti-IgE (ϵ-chain) antiserum were added to rabbit sensitized buffy coat cells (SBC) or normal buffy coat cells (NBC) in TTBSA, and kept at 37°C for 10 min. The cells were centrifuged, the supernatants dialyzed 24 hr, and 25 μl in 2 ml of TG were treated 10 min at 37°C with 2.5 mg of apyrase. 2.5×10^8 platelets were then added, the tubes were placed in an aggregrometer, and platelet aggregation and histamine release measured as described in the legend to Fig. 4. Supernatants from the incubation of SBC with a nonrelated protein contained no detectable PAF.

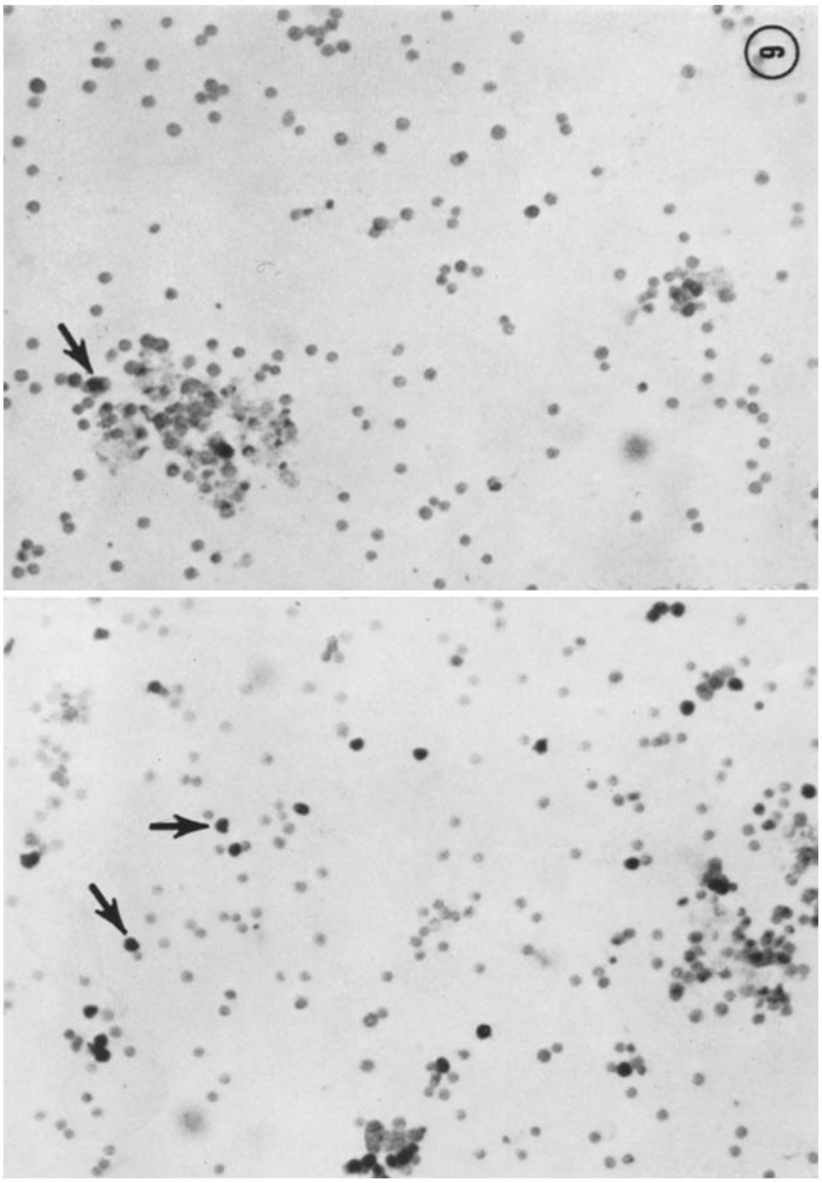


FIG. 9. Preparation of leukocytes from rabbits immunized with peroxidase, stained with toluidine blue, after exposure at 37°C for 19 min to HGG (left) or peroxidase (right). Arrows show brown-stained basophils. The few basophils detectable after reaction with the antigen were partially degranulated.

micrographs by the presence of large black osmiophilic granules. These granules varied not only in number from cell to cell, but also in shape and structure within the same cells. 5 min after addition at 37°C of an unrelated protein to leukocyte preparations from immunized rabbits or of peroxidase to leukocyte preparations from normal rabbits, platelets surrounding the granulated basophils were spatially distinct and morphologically normal; α and dense granules and the canalicular system were well defined (Fig. 10). In contrast, 1–2 min after peroxidase was added to the sensitized leukocytes, basophils began to degranulate and granules appeared to dissolve (Fig. 11). This degranulation

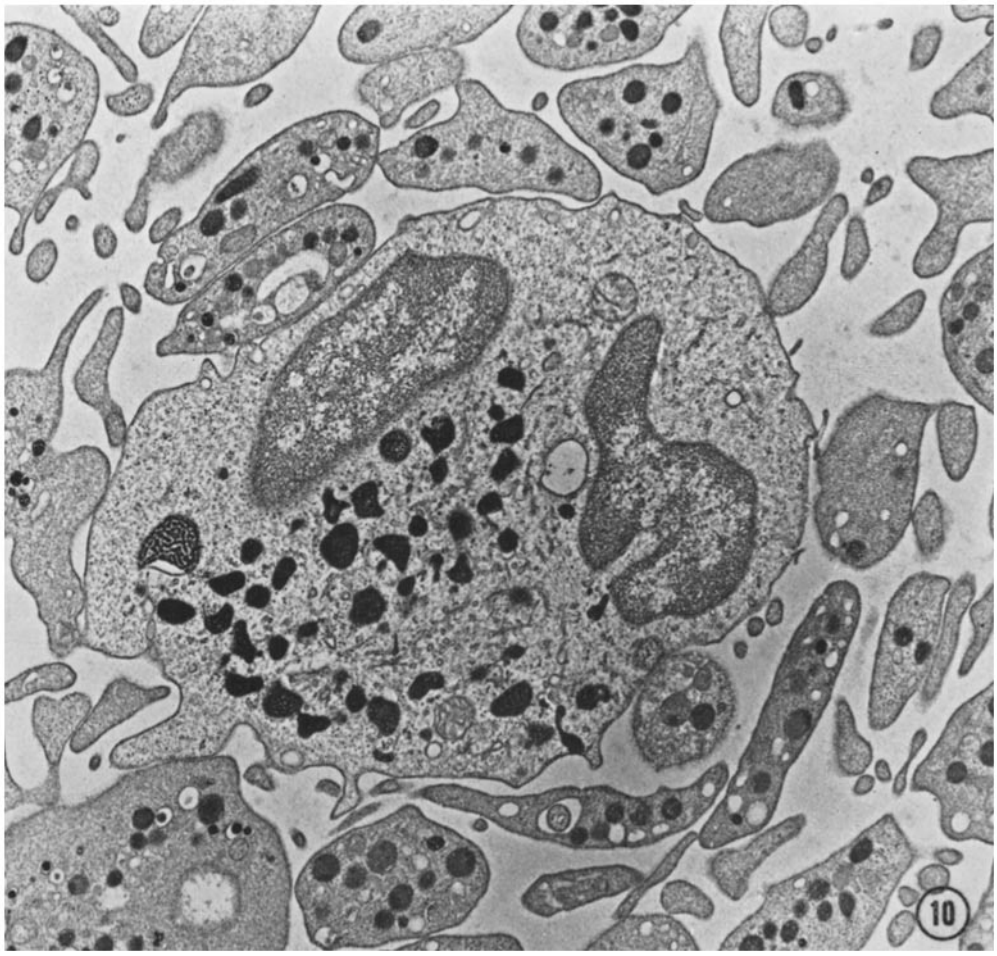


FIG. 10. Normal rabbit basophils, surrounded by normal platelets. Preparation of cells exposed 5 min at 37°C to an unrelated protein. × 12,000.

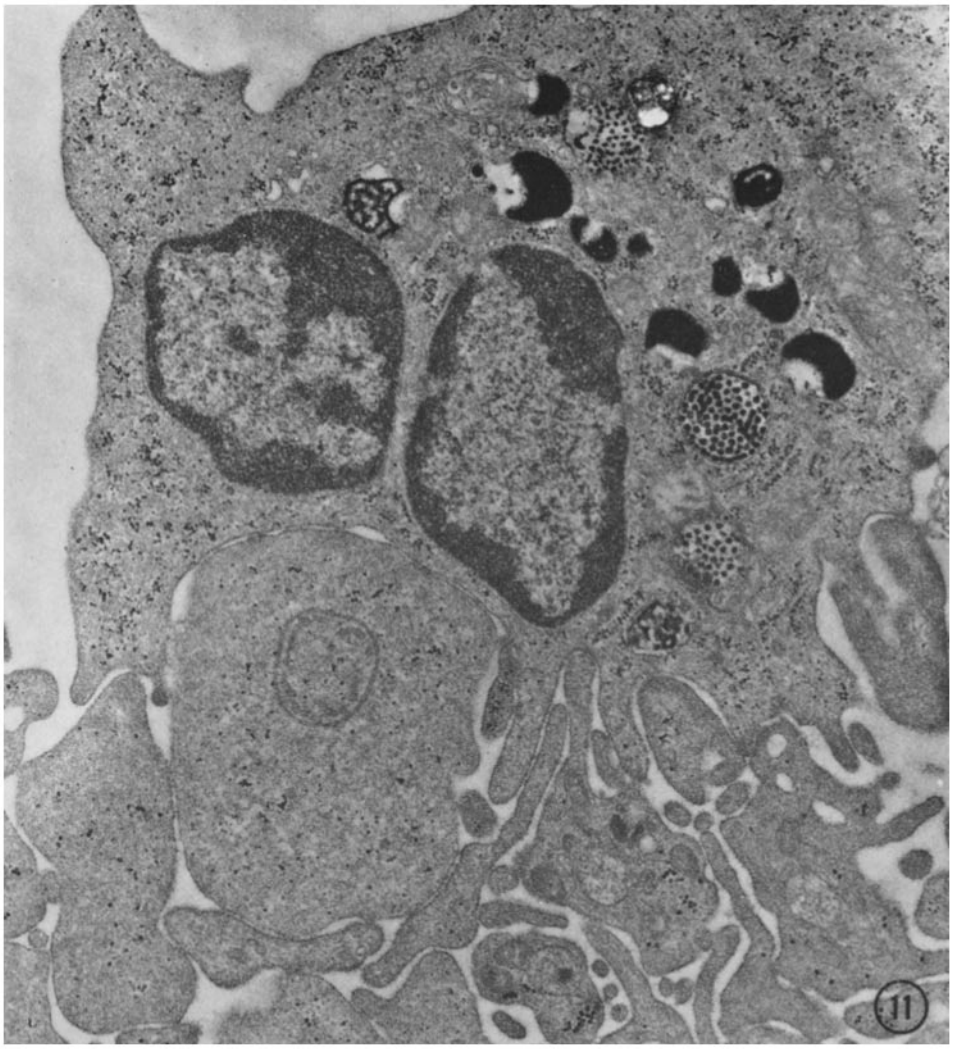
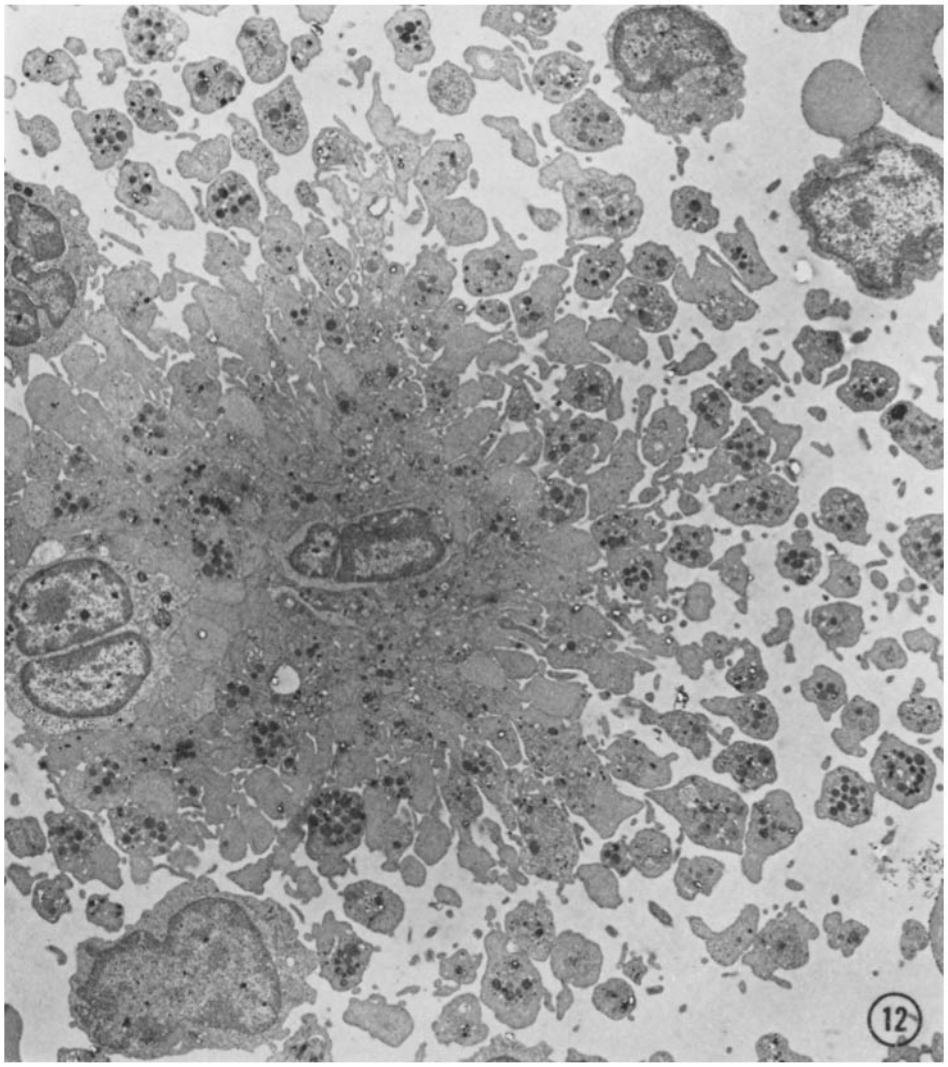
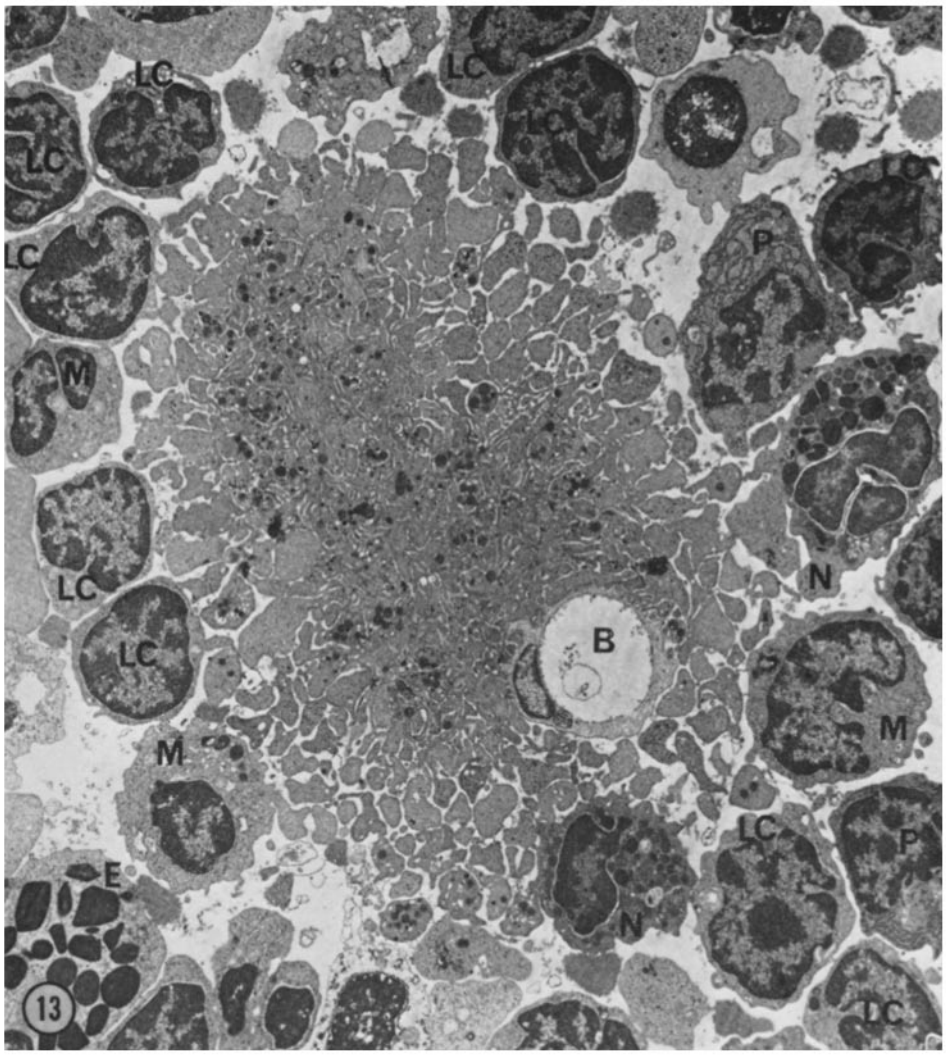


FIG. 11. Preparation of cells from immunized rabbits exposed 2.5 min to peroxidase at 37°C. Different stages of degranulation of the basophil are apparent, while platelets are closely adhering to it; granules and canalicular systems have disappeared in most platelets or appear to be concentrated in the center of the cell. × 22,400.

process of rabbit basophils was totally different from degranulation by extrusion of rat mast cells or neutrophils. The structure of the granules became more apparent as they dissolved. Surrounding platelets aggregated at the same time and their granules and canalicular system disappeared. After 5 min, large aggregates of platelets and basophils were formed (Figs. 12 and 13). At the



FIGS. 12 and 13. 3–5 min after exposure to antigen at 37°C, aggregates of platelets can be found around basophils, identifiable because of the remaining dense granules in their cytoplasm. Platelets close to the degranulated basophils have lost their discoid shape, their cytoplasm looks amorphous, and most granules and canalicular systems have disappeared. No other cells than basophils are involved in the aggregation process. In Fig. 13 are represented numerous lymphocytes (*LC*), plasmocytes (*P*), monocytes (*M*), neutrophils (*N*), and one eosinophil, all distinctly apart from the aggregate, in contrast with the basophil (*B*) which is located in the platelet clump. × 4300.



center of the aggregate, basophils could be recognized primarily because of dense residual osmiophilic granules. Platelets at the periphery of the aggregate appeared normal, but progressively lost their granule content closer to the center of the clump. An intimate relationship appeared to exist between basophils and platelet membranes in all aggregates examined (Fig. 13). Morphologically normal monocytes and neutrophils were found very seldom at the periphery of the aggregate and only in very loose association with platelets. Lymphocytes were never shown to be involved. At 10 min platelets were uniformly aggregated throughout the preparation.

When the experiment was performed in a suspension of buffy coat cells and platelets agitated at 37°C (see Materials and Methods section), aggregation of platelets occurred at random to any cells in the preparation. Aggregates were mostly composed of platelets, but white blood cells were randomly found adherent to other white cells or to platelets.

DISCUSSION

Relationship between Antibody Responsible for the Leukocyte-Dependent Mechanism of Histamine Release from Platelets (LDHR) and IgE Immunoglobulin.—By transferring LDHR reactivity to normal rabbits with serum fractions from sensitized rabbits, evidence was obtained that the antibody responsible for LDHR was of the IgE class. LDHR could be transferred by injecting fractions of antiserum, corresponding to fast γ-globulin, or a molecular weight of about 200,000. The capacity to transfer the LDHR activity was almost totally suppressed by absorbing the antiserum against a specific anti-IgE (anti-ϵ-chain) antiserum before injection into rabbits. Heating of the antiserum reduced its activity. These properties fit closely those described by Zvaifler and Robinson (23) that characterize rabbit homocytotropic (IgE) antibody. Furthermore, pretreatment of sensitized rabbit leukocytes with antibodies to rabbit IgE (but not to γ-chains of IgG) abolished their ability to generate an LDHR. In addition, anti-rabbit IgE antiserum (anti-ϵ-chain) but not anti-rabbit IgG (anti-γ-chain) triggered the reaction between sensitized or normal leukocytes and platelets (LDHR) without addition of antigen.

Correlation between homocytotropic antibody (IgE) production and LDHR has been previously reported (3, 5, 11). Our findings contribute direct evidence that the antibody responsible for LDHR is IgE.

The Nature of the Cells Involved in the LDHR Reaction.—We have shown direct evidence that the cell responsible for LDHR was the basophil. Siraganian and Osler (15) previously found a very good correlation between content of histamine from the leukocytes in a Ficoll gradient and the LDHR capacity of the cells. In our hands, indirect evidence for the involvement of basophils was their massive degranulation in the presence of the antigen and the identification of the antibody at the surface of the cells as that of the IgE type. Based on the association between IgE and basophils in human blood (24), it was reasonable to implicate the basophil in the IgE reaction of LDHR in rabbits.

Using electron microscopy, we were able to visualize the LDHR reaction directly and demonstrate that (*a*) basophils degranulated during LDHR and (*b*) platelets aggregated specifically and rapidly in intimate association with degranulated basophils. In the process, platelets lost their dense granules containing vasoactive amines. In the first 5 min, no cells other than basophils were found to be associated with the platelet aggregate, provided that precautions were taken not to agitate the reaction medium. Between 5 and 10 min

in a nonagitated medium or at any time when the LDHR reaction was performed with agitation, large aggregates were found in which platelets bound to monocytes, neutrophils, basophils, and to other platelets. The most likely explanation of these observations is that the basophils release a factor that diffuses quickly into the medium to bind to leukocytes and platelets, thus creating conditions for diffuse and nonspecific aggregation. This would explain why in the literature the morphology of leukocytes adherent to platelet aggregates varies from that corresponding to the small lymphocyte (14) to that resembling the monocyte (17), which are the cell types predominant in rabbit buffy coat preparations.

Release and Detection of Platelet-Activating Factor (PAF).—PAF was found in the medium after basophils have degranulated under the influence of antigen (peroxidase) or anti-ε-chain antiserum. Specific technical conditions are necessary for recovery of PAF:

(*a*) Cell contaminants must be carefully removed from the buffy coat cell suspension. The amount of PAF recovered was markedly reduced when platelets or granulocytes were present in the buffy coat cell preparation. PAF could be removed from solutions by several absorptions with platelets, normal buffy coat cells, granulocytes, or diatomaceous earth.[4] PAF therefore appears to bind to many cells and probably to charged surfaces.

(*b*) Serum albumin must be present in the solution as a PAF carrier. Our data indicate that PAF was effectively bound to serum albumin (e.g., BSA). In this form, it was stable several months at −20°C. Preliminary results have shown that PAF could be extracted from BSA by using 80% ethanol.[4] It was then dialyzable, was very unstable in aqueous buffer, but remained stable when kept at −0.2°C in ethyl alcohol. These properties show some similarities between PAF and the slow reacting substance of anaphylaxis (SRS-A). SRS-A is a factor released by mast cells of sensitized lung or peritoneum in various species (25), upon challenge by antigen or anti-IgE. It binds to serum albumin, is removed from albumin by extraction in 80°C ethyl alcohol, and is a dialyzable molecule. In opposition to SRS-A, however, PAF does not possess any smooth muscle-contracting activity. On the other hand, SRS-A (furnished to us by Doctors R. Orange and F. Austen) did not aggregate platelets. PAF and SRS-A might be considered as two different factors secreted in similar physiological situations by closely related cell types (mast cells-basophils).

The yield of PAF from sensitized buffy coat cell preparations was, in terms of biological activity, very high. Usually 1×10^7 buffy coat cells containing 5–7% basophils were allowed to react with antigen in 1 ml of TTBSA. 100 μl of the cell-free supernatant was generally sufficient to obtain 70–80% release of histamine from 5×10^8 platelets. Since the relative numbers of leukocytes and platelets in the blood are roughly 1×10^7 and 5×10^8, respectively, it is

[4] Benveniste, J. Manuscript in preparation.

apparent that a considerable amount of PAF is available in the basophils. PAF may have other functions than its action on platelets; its vasopermeability properties are under investigation in our laboratory (9).

Relationship between LDHR and Mechanisms of Immediate Hypersensitivity.—The results of this work show that LDHR is an IgE-mediated mechanism involving sensitized basophils which degranulate in the presence of the antigen. During this process, they release their histamine content and a factor which causes rabbit platelets to aggregate and release their vasoactive amines. The identity of LDHR and the well defined IgE-mediated allergic release of histamine from rabbit or human basophils (26) is therefore obvious; the same antibody (IgE) passively sensitizes some cells (basophils) in the two systems, the same antiserum (anti-IgE) inhibits or, depending upon experimental conditions, induces the reaction. The two systems differ only in the conditions under which experiments are performed, i.e., whether or not rabbit platelets are added to the medium in order to detect the release of PAF by basophils.

Role of LDHR in Deposition of Circulating Immune Complexes.—There is indirect evidence that LDHR is involved in the deposition of immune complexes in acute immune complex disease of rabbits. This deposition was not inhibited by depletion of circulating complement (10, 27) and a good correlation was observed between the presence of LDHR and induction of glomerulonephritis (10). Many observations have linked vasopermeability induced by vasoactive amine release and deposition of immune complexes and other macromolecules in blood vessels (28–34) or even worm expulsion through intestinal mucosae in parasitic infections of rats (35). This suggests that anaphylactic reactions might be involved not only in allergic diseases but might also play a physiologic role in defense mechanisms; they could initiate heightened passage of molecules of different sizes and even cells through vessel walls, glomeruli, and mucosae. In rabbits we have shown that such a mechanism could be found in the IgE-mediated degranulation of basophils and release of histamine from platelets. When a situation exists where antigen-antibody complexes or macromolecules are present in the circulation, this mechanism may trigger the events that lead to increased vascular permeability and deposition of the immune complexes in vessel walls to initiate the histological and pathological features of immune complex disease.

SUMMARY

We have studied the leukocyte-dependent mechanism of histamine release (LDHR) from rabbit platelets, a complement-independent mechanism which has been implicated in the deposition of immune complexes in acute serum sickness of rabbits. It was found by chromatography and passive transfer of serum from immunized rabbits that the antibody responsible for the LDHR was of IgE type. By electron microscope study of the reaction, the leukocyte involved in agglutination of platelets and release of their histamine content

was identified as the basophil. Upon addition of antigen, basophils sensitized with IgE degranulated, released their histamine content and a platelet-activating factor (PAF) that caused aggregation of platelets and release of their histamine. Conditions of preparing and preserving PAF activity and some properties of this factor have been elucidated. LDHR must, therefore, be considered as an immediate hypersensitivity-type mechanism which may link allergic reactions with immunologic disease associated with severe structural injury.

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BIBLIOGRAPHY

1. Henson, P. M., and C. G. Cochrane. 1969. Immunological induction of increased vascular permeability. II. Two mechanisms of histamine release from rabbit platelets involving complement. *J. Exp. Med.* **129:**167.
2. Henson, P. M. 1970. Mechanisms of release of constituents from rabbit platelets by antigen-antibody complexes and complement. II. Interactions with neutrophils. *J. Immunol.* **105:**490.
3. Barbaro, J. F., and N. J. Zvaifler. 1966. Antigen induced histamine release from platelets and rabbits producing homologous PCA antibody. *Proc. Soc. Exp. Biol. Med.* **122:**1245.
4. Schoenbechler, M. J., and E. H. Sadun. 1968. In vitro histamine release from blood cellular elements of rabbits infected with *Schistosoma mansoni*. *Proc. Soc. Exp. Biol. Med.* **127:**601
5. Siraganian, R. P., and A. G. Osler. 1969. Histamine release from sensitized rabbit leukocytes and associated platelet involvement. *J. Allergy.* **43:**167.
6. Barbaro, J. F., and M. J. Schoenbechler. 1970. The nature of the reaction of antigen with lymphocytes from rabbits infected with *Schistosoma mansoni* on the release of histamine from rabbit platelets. *J. Immunol.* **104:**1124.
7. Henson, P. M. 1970. Release of vasoactive amines from rabbit platelets induced by sensitized mononuclear leukocytes and antigen. *J. Exp. Med.* **131:**287.
8. Siraganian, R. P., and A. G. Osler. 1971. Destruction of rabbit platelets in the allergic response of sensitized leukocytes. I. Demonstration of a fluid phase intermediate. *J. Immunol.* **106:**1244.
9. Benveniste, J., and R. Papoian. 1972. Platelet activating factor: its release from human and rabbit leukocytes by a reaginic hypersensitivity mechanism. *Fed. Proc.* **31:**748. (Abstr.)
10. Henson, P. M., and C. G. Cochrane. 1971. Immune complex disease in rabbits. The role of complement and of a leukocyte-dependent release of vasoactive amines from platelets. *J. Exp. Med.* **133:**554.
11. Henson, P. M., and C. G. Cochrane. 1969. Antigen-antibody complexes, platelets

and increased vascular permeability. *In* Cellular and Humoral Mechanisms of Anaphylaxis and Allergy. H. Z. Movat, editor. S. Karger AG., Basel. 129.

12. Colwell, E. J., J. R. Ortaldo, M. J. Schoenbechler, and J. F. Barbaro. 1971. In vivo passive sensitization of normal rabbit leukocytes with sera demonstrating homocytotropic antibody activity. *Int. Arch. Allergy. Appl. Immunol.* **41:**754.
13. Benveniste, J., and P. M. Henson. 1971. Leukocyte-dependent mechanism of histamine release from rabbit platelets: transfer of responsible antibody. *Fed. Proc.* **30:**654. (Abstr.)
14. Aikawa, M., M. J. Schoenbechler, J. F. Barbaro, and E. H. Sadun. 1971. Interaction of rabbit platelets and leukocytes in the release of histamine. Electron microscopic observations. *Am. J. Pathol.* **63:**85.
15. Siraganian, R. P., and A. G. Osler. 1971. Destruction of rabbit platelets in the allergic response of sensitized leukocytes. II. Evidence for basophil involvement. *J. Immunol.* **106:**1252.
16. Aster, R. H., and J. H. Jandl. 1964. Platelet sequestration in man. I. Methods. *J. Clin. Invest.* **43:**843.
17. Ardlie, N. G., M. A. Packham, and J. F. Mustard. 1970. Adenosine diphosphate induced platelet aggregation in suspension of washed rabbit platelets. *Br. J. Haematol.* **19:**7.
18. Fallen, H. J., E. Frei, J. D. Davidson, J. S. Teier, and D. Burk. 1962. Leukocyte preparation from human blood: evaluation of their morphologic and metabolic state. *J. Lab. Clin. Med.* **59:**779.
19. Ovary, Z. 1958. Immediate reaction in the skin of experimental animals provoked by antibody-antigen interaction. *Prog. Allergy.* **5:**459.
20. Zvaifler, N. J., and E. L. Becker. 1966. Rabbit anaphylactic antibody. *J. Exp. Med.* **123:**935.
21. Graham, R. C., Jr., and M. J. Karnovsky. 1966. The early stages of absorption of injected horseradish peroxidase in the proximal tubules of mouse kidney: ultrastructural cytochemistry by a new technique. *J. Histochem. Cytochem.* **14:**291.
22. Straus, W. 1962. Colorimetric investigation of the uptake of an intravenously injected protein (horseradish peroxidase) by rat kidney and effects of competition by egg white. *J. Cell Biol.* **12:**231.
23. Zvaifler, N. J., and J. O. Robinson. 1969. Rabbit homocytotropic antibody. A unique rabbit immunoglobulin analogous to human IgE. *J. Exp. Med.* **130:** 907.
24. Ishizaka, K., H. Tomioka, and T. Ishizaka. 1970. Mechanisms of passive sensitization. I. Presence of IgE and IgG molecules on human leukocytes. *J. Immunol.* **105:**1459.
25. Orange, R. P., and K. F. Austen. 1969. Slow reacting substance of anaphylaxis. *Adv. Immunol.* **10:**105.
26. Ishizaka, K., and T. Ishizaka. 1971. Mechanisms of reaginic hypersensitivity: A review. *Clin. Allerg.* **1:**9.
27. Rhyne, M. B., and F. G. Germuth. 1961. The relationship between serum complement activity and the development of allergic lesions in rabbits. *J. Exp. Med.* **114:**633.

28. Alksne, J. F. 1959. The passage of colloidal particles across the dermal capillary wall under the influence of histamine. *Q. J. Exp. Physiol. Cogn. Med. Sci.* **44**:51.
29. Benacerraf, B., R. T. McCluskey, and D. Patras. 1959. Localization of colloidal substances in vascular endothelium. A mechanism of tissue damage. I. Factors causing the pathologic deposition of colloidal carbon. *Am. J. Pathol.* **35**:75.
30. Majno, G., and G. E. Palade. 1961. Studies on inflammation. I. The effect of histamine and serotonin on vascular permeability. An electron microscopy study. *J. Biophys. Biochem. Cytol.* **11**:571.
31. Cochrane, C. G. 1963. Studies on the localization of circulating antigen-antibody complexes and other macromolecules in vessels. I. Structural studies. *J. Exp. Med.* **118**:489.
32. Cochrane, C. G. 1963. Studies on the localization of circulating antigen-antibody complexes and other macromolecules in vessels. II. Pathogenetic and pharmacodynamic studies. *J. Exp. Med.* **118**:503.
33. Cochrane, C. G., and D. Hawkins. 1968. Studies on circulating immune complexes. III. Factors governing the ability of circulating complexes to localize in blood vessels. *J. Exp. Med.* **127**:137.
34. Kniker, W. T., and C. G. Cochrane. 1968. The localization of circulating immune complexes in experimental serum sickness. The role of vasoactive amines and hydrodynamic forces. *J. Exp. Med.* **127**:119.
35. Murray, M., W. F. H. Jarrett, and F. W. Jennings. 1971. Mast cells and macromolecular leaks in intestinal immunological reactions. The influence of sera of rats infected with *Nippostrongylus Brasiliensis*. *Immunology.* **21**:17.